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Effect of polysaccharides from *Opuntia ficus-indica* (L.) cladodes on the healing of dermal wounds in the rat

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Abstract

In traditional medicine extracts of polysaccharide-containing plants are widely employed for the treatment of skin and epithelium wounds and of mucous membrane irritation. The extracts of *Opuntia ficus-indica* cladodes are used in folk medicine for their antiulcer and wound-healing activities. The present study describes the wound-healing potential of two lyophilized polysaccharide extracts obtained from *O. ficus-indica* (L.) cladodes applied on large full-thickness wounds in the rat. When topically applied for 6 days, polysaccharides with a molecular weight (MW) $> 10^4$ Da from *O. ficus-indica* cladodes induce a beneficial effect on cutaneous repair in this experimental model; in particular the topical application of *O. ficus-indica* extracts on skin lesions accelerates the reepithelization and remodelling phases, also by affecting cell–matrix interactions and by modulating laminin deposition. Furthermore, the wound-healing effect is more marked for polysaccharides with a MW ranging 10^4–10^6 Da than for those with MW $> 10^6$ Da, leading us to suppose that the fine structure of these polysaccharides and thus their particular hygroscopic, rheologic and viscoelastic properties may be essential for the wound-healing promoter activity observed.

Keywords: *Opuntia ficus-indica*; Wound healing; Polysaccharides

Introduction

Polysaccharides from plant extracts are an interesting source of additives for several industries, in particular food and drug industry. Many of these polysaccharides, like those from the Cactaceae family, have been empirically used to modify the rheological properties of some products (Pimienta-Barrios, 1991). In traditional medicine, extracts of polysaccharide-containing plants are widely employed for the treatment of skin and epithelium wounds and of mucous membrane irritation (Bedi and Shenefelt, 2002). However, little is known about the pharmacological properties of natural polysaccharides.

Disruption of tissue integrity initiates a complex series of events (namely wound repair), consisting of a predictable set of responses whatever the initiating agent (surgical excisions or incisions, physical or chemical damage, trauma or infection) or the depth of injury; it involves several steps, including hemostasis, acute inflammation, granulation tissue formation, matrix

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formation, remodelling of connective tissue, collagenization and acquisition of wound strength (Schaffer and Nanney, 1996).

On regards the mode of action of plant polysaccharides as wound-healing promoters, the so-called mucilagineous effect is often claimed; the bioadhesion of polysaccharides to epithelia was demonstrated for some traditionally used herbs, such as *Fucus vesiculosus*, *Calendula officinalis*, *Linum usitatissimum*, *Malva sylvestris* and *Trigonella foenum-graecum* (Schmidgall et al., 2000). Furthermore, the ability of several (sulphated and non-sulphated) polysaccharides to interfere with cell–cell and cell–matrix interaction has been described by several authors. For example, *Sedum telephium* L. fresh leaves and juice are efficacious, when topically applied to painful wounds, burns and eczema, to promote healing and reduce inflammation (Raimondi et al., 2000); this capability is due to non-sulphated polysaccharides contained in them. Topical application of *Aloe vera* gel appears to have a beneficial influence on the various phases of wound healing like fibroplasia, collagen synthesis and maturation and wound contraction (Chithra et al., 1998a, b). The beneficial effects of *A. vera* gel, as well as of other plant extracts (e.g., from *S. telephium* leaves or *Paeonia lactiflora* root), in promoting wound healing have been ascribed also to the immunomodulatory capability of oligo- and polysaccharides contained in them (Strickland, 2001; Sendl et al., 1993; Tomoda et al., 1994). Finally, the clinical efficacy of hyaluronic acid may be attributed to a multifaceted role in the mediation of the tissue repair process (Chen and Abatangelo, 1999).

O. ficus indica (L.), a member of the Cactaceae family, is a tropical or subtropical plant originally grown in South America and is cultivated in dry regions as an important nutrient and food source (El Kossori et al., 1998; Lee et al., 2002). The extracts of *O. ficus indica* cladodes are used in folk medicine for their antiulcer and wound-healing activities (Galati et al., 2001; Park and Chun, 2001). The major components of cladodes are carbohydrate-containing polymers, which consists of a mixture of mucilage and pectin. The mucilage contains a molecular structure of up to 30,000 different sugars; a molecular weight (MW) of 2.3×10^{14} was estimed by Medina-Torres et al., (2000), although a higher MW (4.3×10^{6}) has been determined by other authors (Trachtenberg and Mayer, 1982).

The present study describes the wound-healing potential of two lyophilized polysaccharide extracts obtained from *O. ficus-indica* (L.) cladodes applied on large full-thickness wounds in the rat. In particular, we have evaluated the effect of these two extracts on the deposition of the laminin, a key step in the complex series of events resulting in wound repair.

Materials and methods

Drugs

Biopsy punches were bought from Stiefel Laboratories SRL (Milan, Italy). Hill Top Chambers were obtained from Hill Top Research (Cincinnati, OH, USA). Hyaluronic acid sodium salt was purchased from Fluka (Milan, Italy), 10% buffered formalin and 4% paraformaldehyde (Immunofix®) from Bio-Optica (Milan, Italy), polyclonal rabbit anti-laminin antibody and IgG-peroxidase conjugate from Sigma (St. Louis, MO, USA).

Preparation of polysaccharide extracts

Young terminal cladodes of *O. ficus-indica* (L.) were obtained from a commercial plantation in San Cono (Catania, Sicily), in January 2000. Cladodes were washed in water, cut into pieces (10 cm^2), without removing the small leaves and thorns, and then pressed using a hand press. The resulting juice was centrifuged for 15 min at 5000 g using a Beckman Model J2-21 centrifuge. The supernatant (yield 6.1%) was ultrafiltered using a Labscale TFF system (Millipore, Bioprocess Division; Milan, Italy) equipped with three ultrafiltration (UF) membranes (type Pellicon XL, mod. Biomax 10 K; contact area: 150 cm^2) and with a peristaltic pump Millipore. This UF process gave us two fractions constituted, respectively, of polysaccharides with a MW higher (fraction A) and lower (fraction B) than 10^4 Da. Then fraction A was ultrafiltered using the system described above, equipped with three UF membranes (type Pellicon XL, mod. Biomax 1000 K; contact area: 150 cm^2), so as to obtain two fractions containing polysaccharides with a MW $> 10^6$ Da (fraction C) and with a MW ranging 10^4–10^6 Da (fraction D), respectively. Finally fractions C and D were lyophilized with a yield of 0.01% and 0.03%, respectively.

The optical rotation (measured in a Perkin-Elmer 141 Polarimeter by using a 0.1% polysaccharide aqueous solution) of fractions C and D was: $[\alpha]_D^{20} = +60^\circ$ for fraction C and $[\alpha]_D^{20} = +78.2^\circ$ for fraction D. Sugar composition of fractions C and D was analyzed by the methylation method (McGarvie and Parolis, 1981), confirming the presence of arabinose, xylose, galactose, rhamnose and galacturonic acid. The total amount of monosaccharides contained in fractions C and D was determined by a "two-step" process consisting in an initial hydrolysis of polysaccharidic structures and in the quantification of monosaccharide content (expressed as rhamnose) in the hydrolysed fractions by the phenol–sulphuric acid test (Dubois et al., 1956; Mankarios et al., 1979). The fractions C and D contain a 62% (w/w) and a 7.2% (w/w) of monosaccharides, respectively.

Animals

Male Sprague–Dawley rats, 300–400 g (Charles River, Como, Italy), were employed. The animals were housed under standard conditions with free access to food and water. At the moment of the experiment, the animals were divided into two groups, each of six subjects; one group was employed to evaluate the effect of fraction C, the other one to test the effect of fraction D.

Experimental design

On the day of the experiment, the rats were anaesthetized with ether and their back was shaved. Three circular wounds were created for each animal in the dorsal interscapular region by excising the skin with the help of a 6 mm biopsy punch (Basak et al., 2002; Tramontina et al., 2002). One of the three sites was applied with 200 µl of saline solution containing 10% (w/v) of fraction C or fraction D; another site was treated with 200 µl of saline solution containing 10% (w/v) of hyaluronic acid; the third site was used as control, by applying the same volume of the vehicle alone (saline solution). The three sites were then covered with occlusive chambers. The applications were repeated once a day for 6 consecutive days. All the rats were anaesthetized with ether and sacrificed 24 h after the last application. The three wounds were excised, together with a fourth skin specimen (untreated sample), by using a 8 mm biopsy punch; all tissue specimens were preserved in 10% buffered formalin.

Histological and histochemical studies

Tissue specimens were rinsed in 0.2 M phosphate-buffered saline (PBS, pH 7.4) before fixation with 4% paraformaldehyde, processed routinely into paraplast wax and cut to obtain 5 µm thick sections.

For histological examination, the sections were stained with hematoxylin and eosin (H&E) for light microscopy.

For histochemical studies, the sections were incubated for 12 h in a humid chamber with polyclonal rabbit anti-laminin antibody (dilution 1:30). They were subsequently washed in PBS and incubated for 1 h with a goat anti-rabbit IgG-peroxidase conjugate (dilution 1:100). Peroxidase activity was visualized by incubating the sections for 5–10 min in a solution of 0.015% 3,3′-diaminobenzidine in 0.01 M Tris buffer, pH 7.6, added with 0.005% H_2O_2. For negative controls the primary antibody was omitted or substituted by non-immune rabbit serum.

Micrographs were obtained with an Axiophot Zeiss Light Microscope.

Results

Histological observations of the wounds

Histological and histochemical examination of the wounds was performed 7 days after wounding.

In control samples, the wounds exhibited the formation of an eschar covering a thick granulation tissue and no regenerating epithelium was evident (Fig. 1a).

In wounds treated with hyaluronic acid, keratinocytes appeared organized in few layers under a large and thick eschar. Some basal keratinocytes appeared to be vacuolized and dilated intercellular spaces could be observed. The neodermis was well organized and infiltrating cells were poorly represented (Fig. 1b).

Wounds treated with fraction C showed conspicuous granulation tissue and formation of a stratified epithelium. Migrating epithelium from the wound margins was present at different points. Buds of epidermal appendages (hair follicles and sweat glands) were scattered in a dense and homogeneous dermis (Fig. 1c). Skin samples from wounds treated with fraction D showed completed reepithelization under the eschar. Keratinocytes appeared to be normally organized in layers although stratum granulosum was poorly evident. The neodermis showed a typical configuration with scarse infiltrating cells and frequent small vessels (Fig. 1d).

Furthermore, skin wounds treated with fraction C presented a better healing than wounds treated with fraction D; in fact, they appear to epithelialize faster and the rate of wound contraction is higher as compared to wounds treated with fraction D, where macrophages, fibroblasts and neovasculature in the granulation tissue still coexist 7 days after wounding.

When immunostained with anti-laminin antibody, the skin basement membrane in untreated samples presents a normal behaviour, appearing as a continuous line between epidermis and dermis (Figs. 2 and 4).

In control samples, skin wounds show no evidence of epithelial reorganization; in fact, no immunostaining for laminin was observed in keratinocytes and in the basement membrane (Fig. 3a).

Treatment with hyaluronic acid elicited a cutaneous reepithelization, laminin being present in basal keratinocyte cytoplasm and discontinuous in the basement membrane (Fig. 3b).

Skin wounds applied with extract C showed a total reepithelization with a continuous distribution of laminin in the basement membrane (Fig. 3c), similar to that observed in normal skin (Fig. 4). However, in skin wounds treated with extract D, immunostaining for laminin was revealed only in some basal keratinocytes and discontinously in the basement membrane (Fig. 3d).

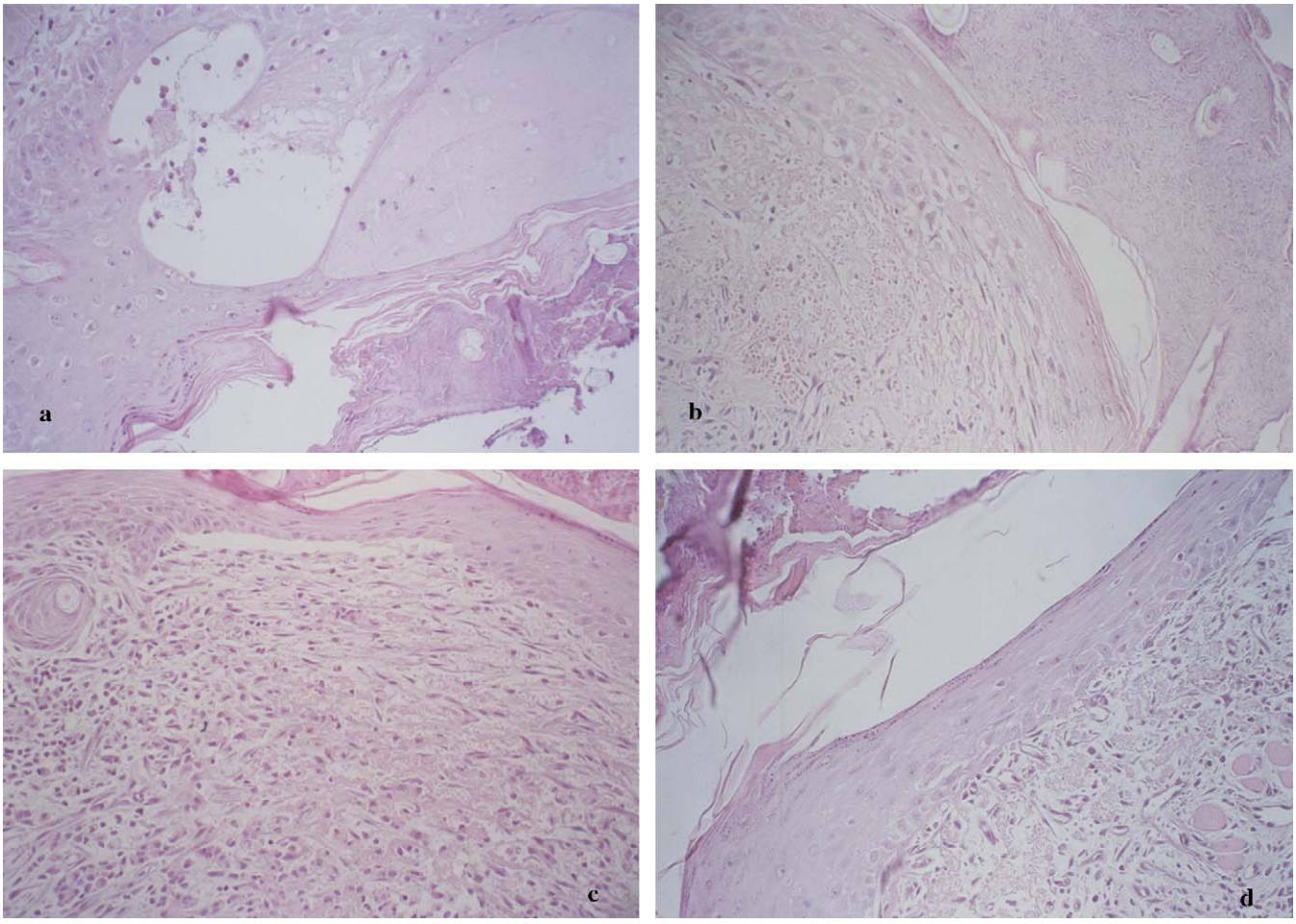

Fig. 1. Histological appearance of the wounded skin treated topically with the vehicle alone (saline solution; control) (a), hyaluronic acid (b), the fraction C (c) and the fraction D (d). (a) Wound shows an eschar and no regenerating epithelium is evident. (b) Regenerating epithelium is organized in few layers of keratinocytes under a thick eschar. Some basal keratinocytes appeared vacuolized. (c) Skin appear well rearranged both epidermis than derma. Note the newly formed granulation tissue accompanied by a stratified epithelium. (d) The re-epithelization is evident under the eschar. The keratinocytes appear normally organized and neodermis shows a typical configuration with new capillary vessels (H&E stain; $\times 20$).

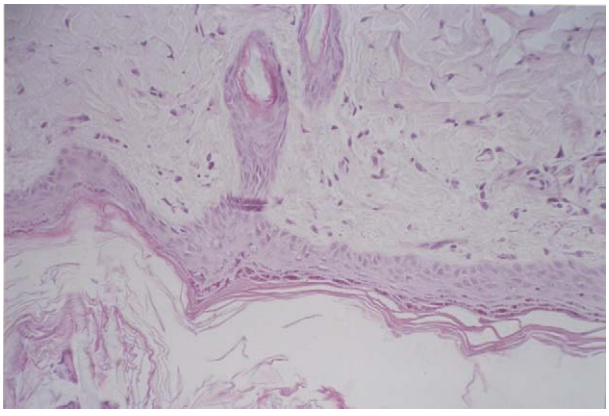

Fig. 2. Normal skin (untreated). The basement membrane appears as a continuous line between epidermis and dermis (H&E stain; $\times 20$).

Discussion

In the present paper we report the wound-healing potential of two lyophilized polysaccharide extracts of *O. ficus-indica* (L.) cladodes applied on large full-thickness wounds in the rat.

Reepithelization plays a crucial role in cutaneous repair, depending upon the specific type of wound (Schaffer and Nanney, 1996; Moulin et al., 2000); it follows a series of tightly regulated, sequential events and occurs in vivo in a variable amount of time.

We observed that the topical application of fractions C and D from *O. ficus-indica* cladodes enhances cutaneous healing, which appeared completed in 7 days. Our histological findings showed that the original tissue regeneration is much greater in skin wounds treated

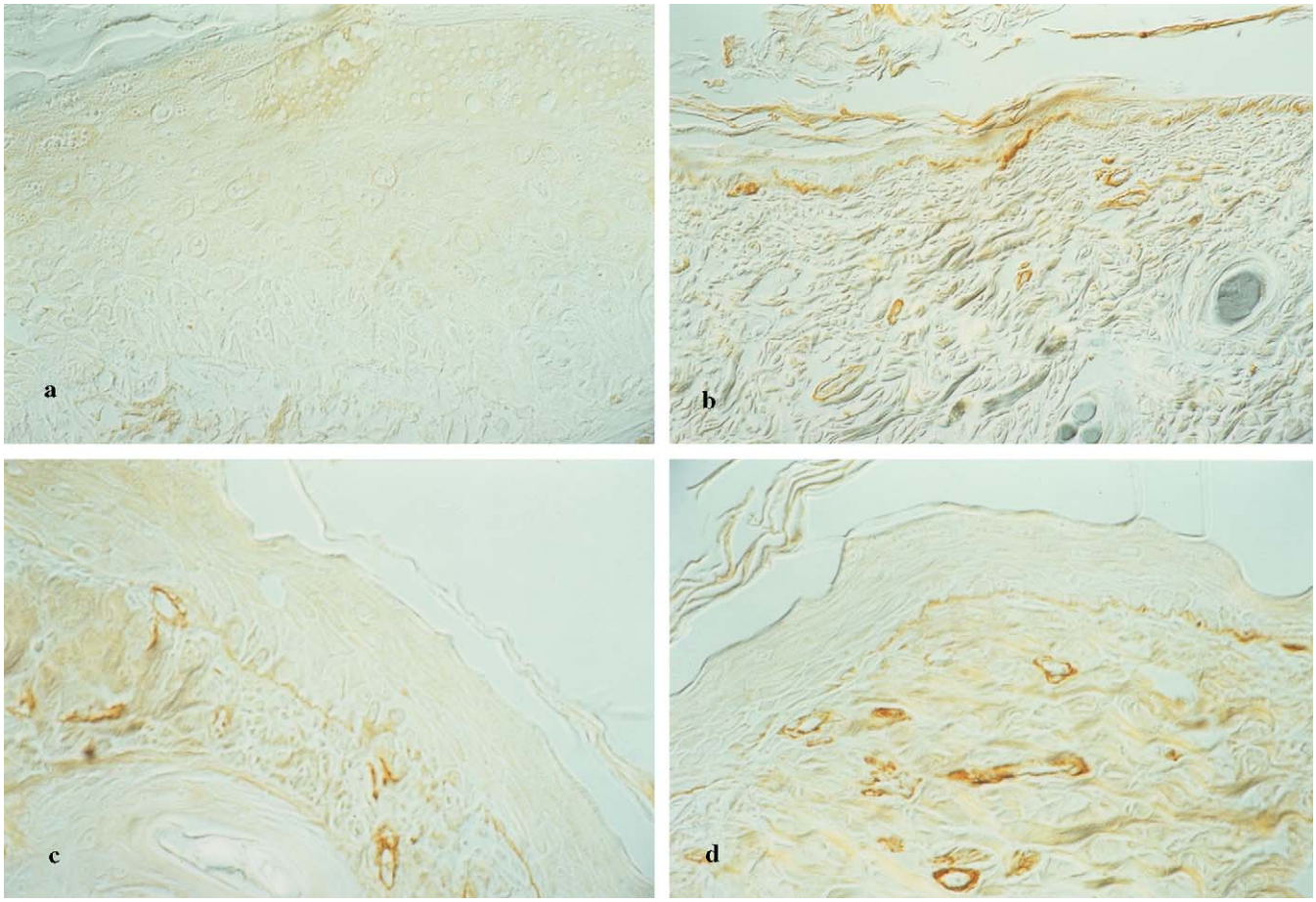

Fig. 3. Immunoperoxidase micrographs of laminin in the wounded skin treated topically with the vehicle alone (saline solution; control) (a), hyaluronic acid (b), the fraction C (c) and the fraction D (d). (a) Wound reveals that there is no laminin in keratinocytes and in the basement membrane. (b) The laminin appears in basal keratinocytes and also in basement membrane. (c) Wound shows a continuous and evident distribution of laminin in the basement membrane; (d) The laminin stains some basal keratinocytes and the basement membrane discontinuously (× 20).

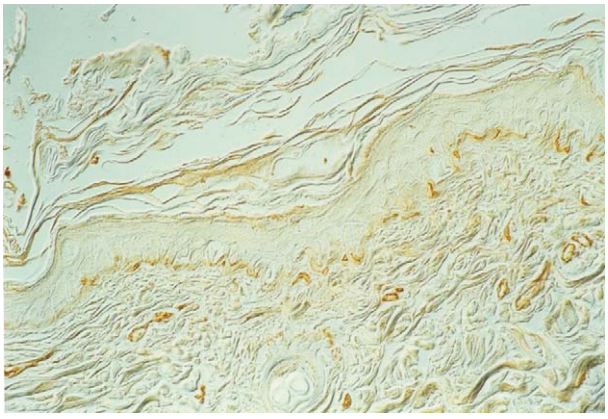

Fig. 4. Immunoperoxidase micrographs of laminin in the normal skin. A continuous distribution of the laminin in the basement membrane is evident (× 20).

with the extracts than in control wounds. In fact, a complete cutaneous repair consisting in neodermis formation and restoration of epidermal layers was observed in Opuntia-treated wounds, while vehicle-treated wounds, at the same time, showed still a thick granulation tissue and no epidermis production.

Furthermore, in skin wounds treated with fractions C and D from *O. ficus-indica* cladodes an improvement of epidermal organization is evident, when compared to the wounds treated with hyaluronic acid, where vacuolized cells in epidermal layers are found. This is very interesting because topical application of hyaluronic acid, as well as of other endogenous glycosaminoglycans (heparin, heparin sulphate, dermatan sulphate), is widely used for the treatment of skin wounds due to their well-known tissue repair and reepithelializing effects (Saliba, 2001).

A good reepithelization induced by the application of the *Opuntia* extracts tested is demonstrated also by the observed deposition of laminin in the skin basement membrane, while laminin is not present in keratinocytes and in the basement membrane zone of control samples.

Following injury disrupting skin basement membrane, the epidermal cells lose their contact with it,

and come in contact with the dermis. Under these conditions, the epithelial cells modify their behaviour and switch from a quiescent phenotype to a migratory one and cover and close the wound (Nishiyama et al., 2000). The basement membrane contains the anchoring complex which maintains the adherence of epidermis to dermis in normal skin (Nishiyama et al., 1998; Mondello et al., 1996); key components of this attachment complex are the laminins, heterotrimers of alpha, beta, and gamma chains, which play important roles in the attachment of cells to the basement membrane (Malinda and Kleinman, 1996; Magaudda et al., 1997) and also in migration, proliferation and differentiation of epithelial cells (Sato et al., 1999).

In fact, the laminin has functional domains which can bind integrins of basal keratinocytes and type VII collagen, forming anchoring fibrils, essential to the resistance of the epidermis. Laminin also accelerates the assembly of basement membranes at the dermal–epidermal junction and its deposition can instruct keratinocytes to switch from an activated phenotype to a quiescent and integrated epithelial phenotype (Nguyen et al., 2000). Once the basement membrane has been assembled, epidermal cells recognize the surface adjacent to the basement membrane as the basal surface. Stratification of the epidermis proceeds, with the proliferating cells remaining attached to the basement membrane and the daughter cells migrating into the upper layers (Nishiyama et al., 2000).

Finally, skin wounds treated with fraction C presented a better healing than wounds treated with fraction D. The reduction of the neovasculature and the organization of collagen fibrils in dermis observed in our tissue specimens suggest that fraction C accelerates, more than fraction D, the process of remodelling, essential for a quick wound repair. During this phase of eschar formation the stimulation of collagen synthesis is known to exceed that of extracellular collagen degradation so that the total amount of collagen continues to increase, and collagen fibrils become tightly packed and stabilized by the formation of inter- and intra-molecular cross-links (Mutsaers et al., 1997). Furthermore, following fraction D application a more complete laminin assemblage in the shin basement membrane is observed than after fraction C application, confirming a faster reepithelization process induced by fraction D.

In conclusion, this report attests that polysaccharides with a MW $>10^4$ Da from *O. ficus-indica* cladodes induce a beneficial effect on cutaneous repair of large full-thickness wounds in the rat. Our findings do not allow us to determine exactly the biological process involved in wound healing if influenced by these *O. ficus-indica* extracts and by which mechanism. On the other hand, the function of the polysaccharide extracts tested is very likely complex and difficult to be specifically attributed to any single one of their properties. However, it can be speculated that the topical application of *O. ficus-indica* extracts on skin lesions accelerates the reepithelization and remodelling phases, also by affecting cell–matrix interactions and by modulating laminin deposition. Interestingly, topical ointments containing extracts of cladodes of wild *Opuntia* plants are widely used in folk medicine as wound-healing promoters, so demonstrating the feasibility to obtain topical formulations containing purified polysaccharides from *O. ficus-indica* cladodes to be employed in human therapy; furthermore the rheological properties of gels containing *O. ficus-indica* mucilage have been extensively investigated (Medina-Torres et al., 2000, 2003).

One has to point out that the wound healing effect is more marked for polysaccharides with a MW ranging 10^4–10^6 Da than for those with MW $>10^6$ Da. One can suppose that the fine structure of these polysaccharides and thus their particular hygroscopic, rheologic and viscoelastic properties (Medina-Torres et al., 2003) are essential for the observed wound-healing promoter activity; further investigations are warranted to investigate their structure and thus to elucidate a clear structure–effect relationship.

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